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YAMAMOTO et al.

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FOR: CHEMOTHERAPEUTIC AGENT-INCORPORATED PHARMACEUTICAL  
PREPARATION

\* \* \*

**REQUEST TO REPUBLISH CORRECTED PATENT APPLICATION PUBLICATION**

February 13, 2008

**Mail Stop PGPub**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

This application was published on July 27, 2006 as US 2006/0165656 A1. It contains a material mistake which is apparent from Office records: i.e., the proper Abstract of the Disclosure is missing. The published Abstract of the Disclosure appears to be for a different patent application. Therefore, correct the published (57) Abstract by inserting the Abstract of the Disclosure which was submitted in this patent application.

This is a material mistake because it at least affects the public's ability to appreciate the technical disclosure of the patent application publication and determine the scope of the patent application publication. This request is not filed within two months from the date of the patent application publication.

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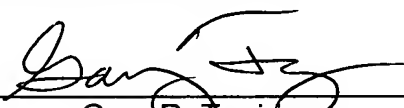
Attached is a copy of the patent application publication with the error clearly marked. See Attachment A. The Abstract of the Disclosure was correctly submitted as shown by reference to the Abstract filed April 6, 2005 (see Image File Wrapper for document code ABST). See Attachment B.

A copy of the application in compliance with the EFS requirements is attached. See Attachment C. The required publication and processing fees due under 37 CFR §§ 1.18(d) and 1.17(i), respectively, are also attached. If the fees are missing or deficient, the Commissioner is authorized to debit our Deposit Account No. 14-1140, under Order No. 423-70.

Do not hesitate to contact the undersigned if additional information is required. Prompt republication of this patent application with the correct Abstract of the Disclosure is requested.

Respectfully submitted,

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(19) **United States**(12) **Patent Application Publication** (10) Pub. No.: **US 2006/0165656 A1****Yamamoto et al.**(43) Pub. Date: **Jul. 27, 2006**(54) **MEDICINAL PREPARATION HAVING  
CHEMOTHERAPEUTIC ENCAPSULATED  
THEREIN****Publication Classification**(76) Inventors: **Seiji Yamamoto, Suita-shi Osaka (JP);  
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514/449; 514/211.08; 435/456**(21) Appl. No.: **10/530,473**(22) PCT Filed: **Oct. 29, 2003**(86) PCT No.: **PCT/JP03/13860**(30) **Foreign Application Priority Data**

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(57) **ABSTRACT**

An apparatus and a method of remote control which can enable real time operation of a device in home from a terminal device at remote location through a network are provided. A first server communicates with a terminal device through the Internet and generates device control data for controlling the device. A second server communicates with the device in a predetermined manner to acquire and store a specific address of the device, generates transmission data for transmission of the received device control data from the first server to the device based on the specific address, and transmits the transmission data to the device. This allows real time control of the device from the terminal device through the network to be realized.

(57) **Abstract:** It is intended to provide a medicinal preparation for transferring a chemotherapeutic (preferably an anticancer agent) into cells or the living body by using a virus envelope vector. Namely, a medicinal preparation containing a virus envelope vector having a chemotherapeutic encapsulated therein as the active ingredient. Examples of the anticancer agent include bleomycins, anthraquinone carcinostatic agents, mitomycins, actinomycins, taxane derivatives, camptothecins, cisplatin, staurosporines, vincristine, streptozotocin, 5-fluorouracil (5-FU) and its derivatives, pirarubicin, dolastatin and pharmacologically acceptable salts thereof. Examples of the virus include sendai virus, retro virus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, pox virus, influenza virus and so on.

Fig. 1

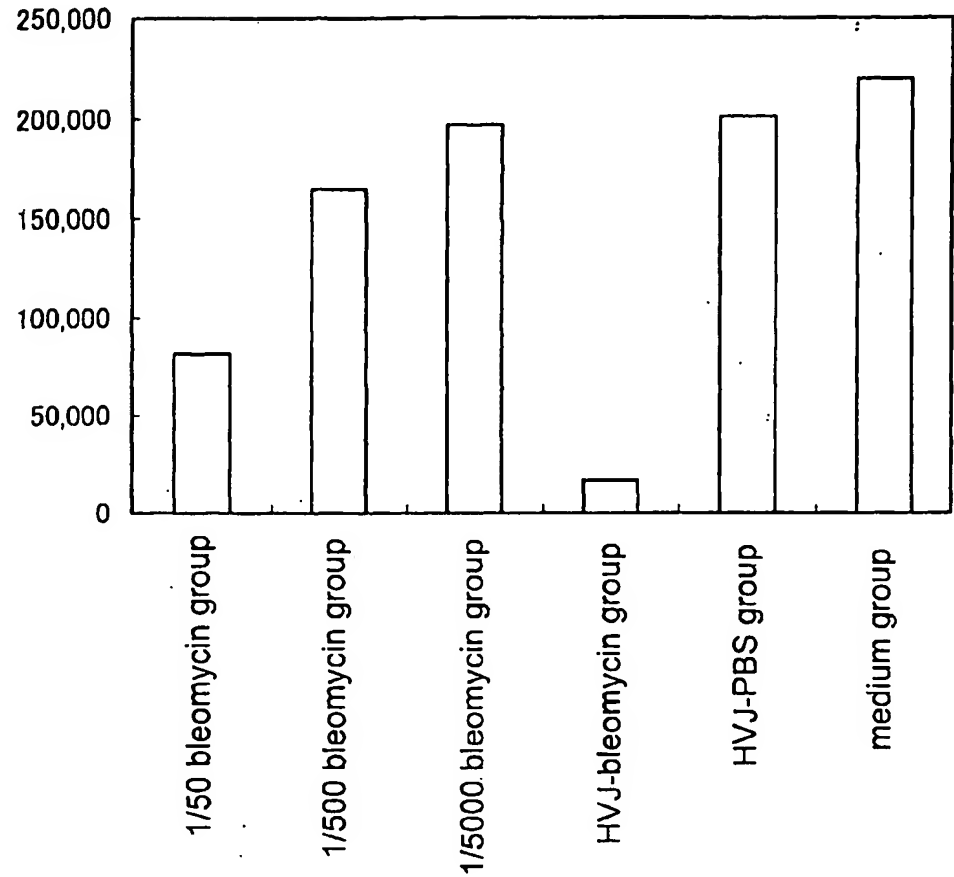


Fig. 2

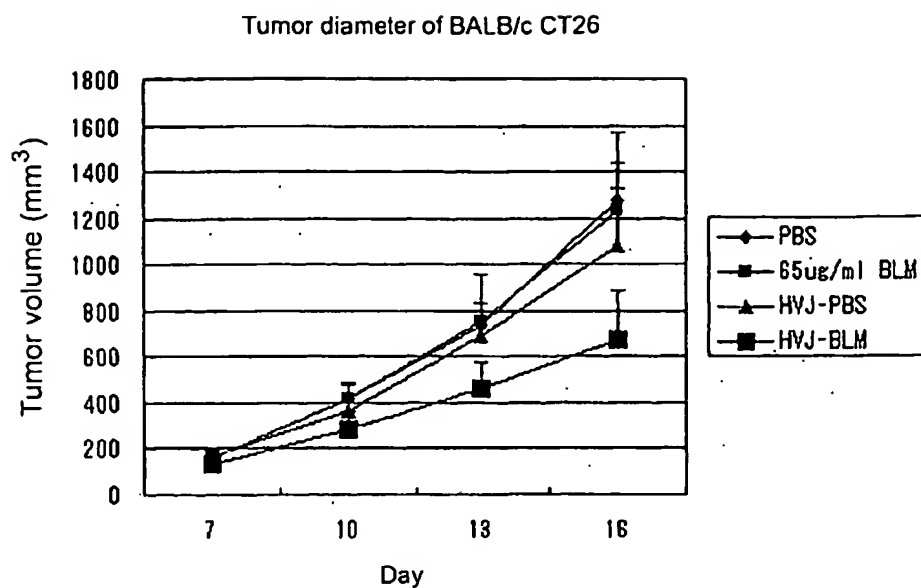


Fig. 3

Average tumor volume in each group on Day 16 after administration

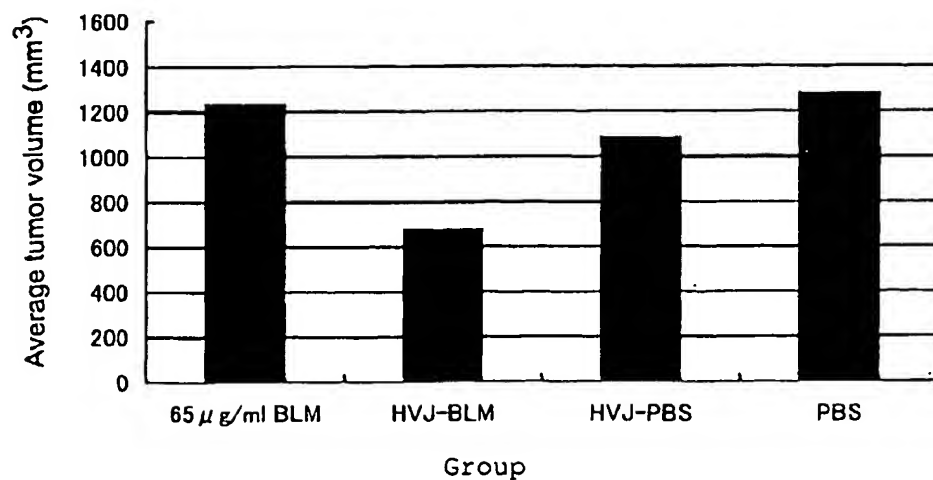
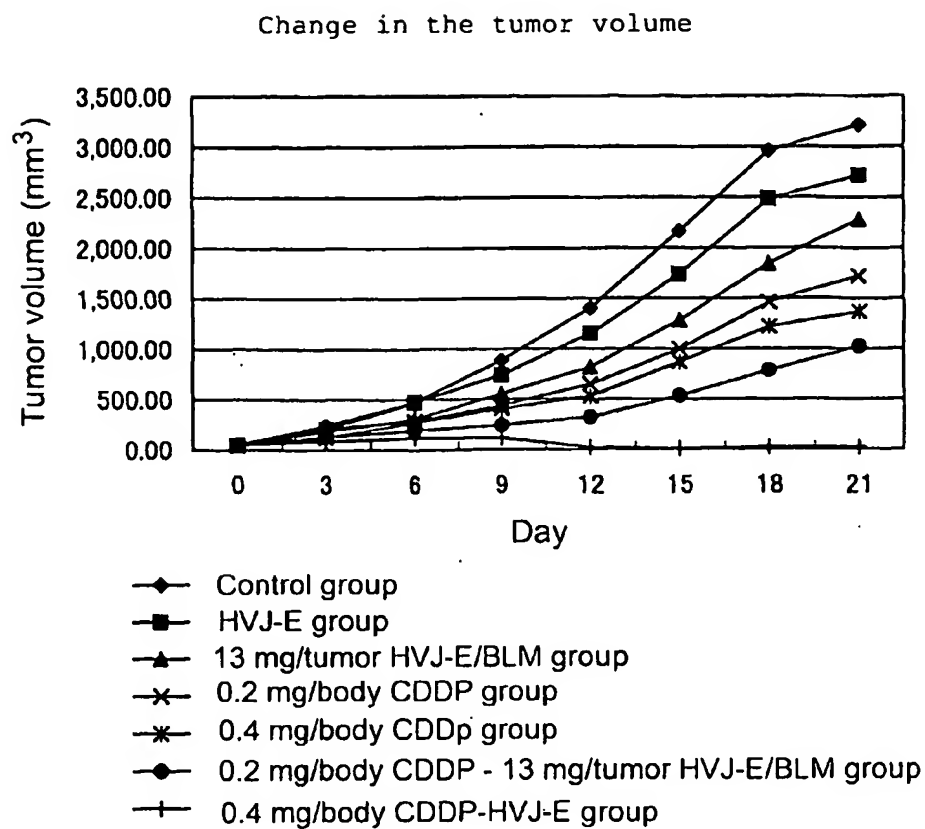


Fig. 4



# MEDICINAL PREPARATION HAVING CHEMOTHERAPEUTIC ENCAPSULATED THEREIN

## TECHNICAL FIELD

[0001] The present invention relates to a pharmaceutical preparation used in transfection of cells or living organisms with a chemotherapeutic agent by a gene delivery vector.

## BACKGROUND ART

[0002] It is said that the cure rate in cancer treatment is about 50%. at present, and generally, such cure is brought about often by topical therapy such as surgical therapy and radiotherapy. It is in a very low rate that chemotherapy as systemic therapy can contribute solely to cure, particularly in treatment of solid tumor, and usually chemotherapy is used in combination with various therapies.

[0003] On the other hand, surgical therapy enables surgery in every organ cancer and is considered to reach completion as therapy, and no further improvement in cure rate can be expected. The treatment results of susceptible organ cancers by radiotherapy also arrive at an almost fixed rate, and no further improvement in cure rate can be expected as well.

[0004] Accordingly, no significant improvement in cancer cure rate by these therapies can be expected in the future, and the development of further excellent chemotherapy is essential for further improving the cancer cure rate of 50% at present to arrive at cure for cancer.

[0005] An purpose of an anticancer agent used in chemotherapy lies in cytotoxic effect on cells having a high ability to grow, such as cancer cells, and its damage to normal cells particularly myeloid cells having a high cellular growth ability is significant, and as a result, severe pain is given to patients. This is because the transfer of the anticancer agent is due to systemic administration by an injection, and the anticancer agent reaches normal cells other than cancer cells, so that the normal cells are killed and homeostasis does not function.

[0006] At present, however, the effect of an anticancer agent administered alone is regarded to be approximately about 30%, and it is expected that genetic information analysis and study on genome proceed so that selection of a suitable anticancer agent feasible can be expected in the future, but it is said that the therapy with the anticancer agent at present results in higher side effects.

[0007] This is because normal cells are damaged by systemic administration of an anticancer agent. Accordingly, if cancer tissue-specific delivery of the anticancer agent and subsequent incorporation thereof into cancer cells can be established, an ideal system of delivering the anticancer agent can be realized. In addition, if incorporation of the anticancer agent into a vesicle is feasible, a therapeutic method that is specific for target organ and cell with less influence (side effect) on normal cells can be established. Further, this can lead to reassessment of anticancer agents whose development was abandoned because of their strong side effects.

## DISCLOSURE OF THE INVENTION

[0008] As a result of extensive study for solving the problem described above, the present inventors could com-

plete a pharmaceutical preparation comprising, as an active ingredient, a virus envelope vector having a chemotherapeutic agent incorporated therein.

[0009] Accordingly, the present invention provides, for example, a pharmaceutical preparation comprising an anticancer agent or the like incorporated in e.g. an inactivated HVJ-E vector having an ability to incorporate a foreign gene.

[0010] The present invention relates to a pharmaceutical preparation used in transfection of cells or living organisms with a chemotherapeutic agent, preferably a cancerocidal agent, an anticancer agent, or an antitumor agent (hereinafter referred to collectively as anticancer agent), by using a gene delivery vector. The present invention relates more specifically to a pharmaceutical preparation by which a highly toxic anticancer agent is transferred by a gene delivery vector to the living organism and allowed to reach a target organ or cell safely with a reduction in side effects.

## BEST MODE FOR CARRYING OUT THE INVENTION

[0011] Hereinafter, the present invention is described in more detail.

[0012] The chemotherapeutic agent used in the present invention is not particularly limited insofar as it is a low-molecular compound acting directly on cells, and for example, "Seikagaku Jiten" (Dictionary of Biochemistry), third edition, published by Tokyo Kagaku Dojin, describes: "At present, the subject of chemotherapy, that is, therapy using chemical substances of highly selective toxicity, has spread not only to microbial infections but also to malignant tumors", and it goes without saying that antibacterial agents, antitumor agents etc. are contained in the chemotherapeutic agent.

[0013] Preferable examples of the chemotherapeutic agent in the present invention include a cancerocidal agent, an anticancer agent or an antitumor agent (hereinafter referred to collectively as anticancer agent), and specifically the anticancer agent includes bleomycin and derivatives thereof, anthraquinone-based cancerocidal agents including adriamycin and daunomycin, mitomycin and derivatives thereof, actinomycin and derivatives thereof, taxane derivatives such as taxol, camptothecin and derivatives thereof such as irinotecan, cisplatin and derivatives thereof, staurosporine and derivatives thereof, vincristine, streptozotocin, 5-fluorouracil (5-FU) and derivatives thereof, viralbacin and dolastatin, as well as pharmacologically acceptable salts thereof.

[0014] Preferable among these chemotherapeutic agents are bleomycin and derivatives thereof, and specific examples include bleomycin or pharmacologically acceptable salts thereof, or peplomycin or pharmacologically acceptable salts, more specifically bleomycin hydrochloride, bleomycin sulfate and peplomycin sulfate.

[0015] When the pharmaceutical preparation of the present invention is used as an anticancer agent, the type of cancer to which it is applicable is not particularly limited, and specifically, solid cancer, blood cell cancer, and the like can be exemplified. Among these cancers, the solid cancer is a preferable subject to which the pharmaceutical preparation is applicable.

[0016] Specific examples of the solid cancer include lung cancer, breast cancer, digestive organ cancer, head and neck cancer, gynecologic cancer, urologic cancer, soft tissue and bone sarcoma, malignant lymphoma, cancers of unknown primary etc., and more specifically, for digestive organ cancers, stomach cancer, colon cancer, and esophagus cancer are exemplified, for head and neck cancers, upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, larynx cancer, and oral cavity cancer are exemplified, for gynecologic cancers, uterus cancer, ovarian cancer, and uterine cervical cancer are exemplified, and for urologic cancers, prostate cancer is exemplified.

[0017] Among these solid cancers, more preferable subjects include skin cancer, skin malignant tumor, head and neck cancers (upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, oral cavity cancer, and the like), lung cancer (particularly primary and metastasis flat epithelial cancer), esophagus cancer, malignant lymphomas (reticulum sarcoma, lymphosarcoma, Hodgkin's disease, and the like), uterine cervical cancer, neuroglioma, thyroid cancer, and prostate cancer.

[0018] The virus envelope vector in the present invention is a virus envelope which is prepared by removing RNA or DNA from virus. It is usually utilized to incorporate a gene, polynucleotide, oligonucleotide, plasmid or the like thereinto, for transfection.

[0019] The type of the virus is not limited, and specifically the virus includes, for example, viruses belonging to a family selected from the group consisting of the retrovirus family, togavirus family, coronavirus family, flavivirus family, paramyxovirus family, orthomyxovirus family, bunyavirus family, rhabdovirus family, poxvirus family, herpes virus family, baculovirus family, and hepadna virus family.

[0020] Specifically, the virus in the present invention includes, for example, Sendai virus, retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poxvirus, influenza virus, and the like.

[0021] Among these viruses, Sendai virus (hemagglutinating virus of Japan (referred to hereinafter as HVJ)) that is one of mouse pneumonia viruses can be mentioned as a preferable example.

[0022] Specifically, Sendai virus, for example, VR-105 and VR-907 can be purchased from American Type Culture Collection (ATCC), telephone 1-703-365-2700, P.O. Box 1549, Manassas, Va. 20108, USA,

[0023] <http://www.atcc.org/SearchCatalogs/long-view.cfm?view=av,152> 376,VR-105&text=Sendai&max=20,

[0024] <http://www.atcc.org/SearchCatalogs/long-view.cfm?view=av,137> 5478,VR-907&text=Sendai&max=20).

[0025] The virus envelope vector is described in more detail in, for example, JP-A 2001-286282 (WO01/57204), JP-A 2002-065278, WO-A 03/014338 (PCT/JP02/07879), and can be prepared specifically according to e.g. Example 8 in JP-A 2001-286282.

[0026] In the step of incorporating a chemotherapeutic agent in the virus envelope vector, a surfactant is preferably used, and specific examples of the surfactant include Triton

X100, deoxycholic acid or a salt thereof, and cholic acid or a salt thereof. Preferably, the salt of deoxycholic acid includes sodium deoxycholate, and preferably, the salt of cholic acid includes sodium cholate.

[0027] The form of the pharmaceutical preparation of the present invention is not limited, but its specific example is an injectable solution, ointment, or the like, preferably an injectable solution.

[0028] Here, the present inventable solution is described in more detail by reference to the inactivated Sendai virus envelope vector (referred to hereinafter as HVJ-E vector).

[0029] When an anticancer agent is incorporated into the HVJ-E vector, the anticancer agent is dissolved in a buffer solution. The buffer solution used herein is not limited, and specifically, for example, TE buffer solution (10 mM Tris, 1 mM EDTA (pH 8.0)), PBS (phosphate buffer solution) can be suitably selected and used wherein the pH of the buffer solution is preferably 6 to 9.

[0030] A preferable feature of the present invention is that, in an in vitro experiment, an anticancer agent having a strong side effect or toxicity can be incorporated into HVJ-E vector to be delivered directly into cells without leakage of the anticancer agent into a culture solution.

[0031] In an in vivo animal experiment, not systemic administration but local administration of the anticancer agent is feasible, and the anticancer agent can be efficiently delivered into solid-cancer cells only.

[0032] Humans can be treated by chemotherapy of administering the anticancer agent-incorporated HVJ-E vector solely, or by locally administering it into progressive cancer patients whom the anticancer agent cannot be administered, to attain cancer regression, and simultaneously using radiotherapy and/or surgical treatment to achieve further excellent anticancer effects.

[0033] In an in vitro experiment, host cells are transfected with the anticancer agent-incorporated HVJ-E vector. As the procedure in this case, for example, a method of adding a solution of the anticancer agent-incorporated HVJ-E vector to a medium for culturing the cells can be used.

[0034] The transfection is performed for about 30 minutes to 48 hours when it is carried out at 37° C. Judgment of the effect is conducted preferably by counting the number of viable cells or by WST assay (technique of counting viable cells; cell counting kit, Dojin Kagaku).

[0035] When the subject of an in vivo animal experiment is, for example, a mouse, it is preferable that a normal mouse that is not an immune-deficient mouse is used where the cancer cell is an isograft, and that a nude mouse or SCID mouse is used in the case of xenograft.

[0036] Cancer cells cultured in a Petri dish are transplanted subcutaneously to a mouse, and after growth of the transplanted cells, the anticancer agent-incorporated HVJ-E vector is administered into the grown solid cancer, and the major axis and minor axis of the cancer can be measured to determine the anticancer effect.

[0037] According to the present invention, there is provided a method capable of delivering an anticancer agent having a strong side effect to the site of a cancer easily and safely.



[0038] Accordingly, the HVJ-E vector can be used to enable new chemotherapy for any solid cancer, which is increasing rapidly in Japan, such as lung cancer, breast cancer, digestive organ cancers such as stomach cancer, colon cancer or esophagus cancer, head and neck cancers (upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, larynx cancer, oral cavity cancer etc.), gynecologic cancers (uterus cancer, ovarian cancer, uterine cervical cancer etc.), urologic cancers (prostate cancer), soft tissue and bone sarcoma, malignant lymphoma, cancers of unknown primary, and the like.

#### BRIEF DESCRIPTION OF DRAWINGS

[0039] FIG. 1 is a graph wherein the numbers (mean $\pm$ standard deviation) of viable cells in the respective groups in an in vitro experiment are compared.

[0040] FIG. 2 is a graph wherein the average tumor volumes (mean $\pm$ standard deviation) in the respective groups in an in vivo experiment are compared.

[0041] FIG. 3 is a graph showing a change in the average tumor volume (mean $\pm$ standard deviation) in each group, relative to the medium (PBS) group, on Day 16 after administration in an in vivo experiment.

[0042] FIG. 4 is a graph showing the result of tumor volume in Example 3.

#### EXAMPLES

[0043] Hereinafter, the present invention is described in more detail by reference to Examples, but the present invention is not limited by these Examples.

##### Example 1

##### In vitro Experiment

[0044] According to Example 8 in JP-A 2001-286282, 6,000 HAU/600  $\mu$ l (for six 6-well plates) of inactivated HVJ-E vector was allowed to have a change in temperature from  $-80^{\circ}$  C. to  $34.5^{\circ}$  C. A microtube containing the sample was centrifuged at 15,000 rpm for 15 minutes at  $4^{\circ}$  C. to give the HVJ-E vector as a precipitate, and the supernatant was removed. The resulting precipitate was suspended in 60  $\mu$ l bleomycin/PBS (5 mg/ml) (bleomycin HCl manufactured by Nippon Kayaku Co., Ltd.) solution. Further, 2  $\mu$ l of 3% Triton-X100 was added thereto to prepare a sample containing Triton-X100 at a final concentration of 0.1%, which was then left on ice for 15 minutes. Thereafter, 500  $\mu$ l PBS solution was added thereto. The microtube was centrifuged at 15,000 rpm for 15 minutes at  $4^{\circ}$  C., and the supernatant was removed without removing the precipitate, and 500  $\mu$ l PBS solution was added again to the precipitate. The microtube was centrifuged again at 15,000 rpm for 15 minutes at  $4^{\circ}$  C., and the supernatant was removed without removing the precipitate.

[0045] The resulting precipitate was suspended in 180  $\mu$ l PBS, and the resulting sample solution was pipetted in a volume of 30  $\mu$ l/microtube into 6 microtubes. Five microliters of protamine sulfate solution prepared at 5 mg/ml and 500  $\mu$ l DMEM solution (Dulbecco's modified Eagle medium) were added to each tube.

[0046] As the administration group, the followings were prepared and compared to evaluate their effect.

[0047] HVJ-bleomycin group; 1,000 HAU, bleomycin 200 ng/DMEM 500  $\mu$ l/well.

[0048] HVJ-PBS group; 1,000 HAU/DMEM 500  $\mu$ l/well.

[0049] 1/50 bleomycin group: bleomycin 50  $\mu$ g/DMEM 500  $\mu$ l/well.

[0050] 1/500 bleomycin group: bleomycin 5  $\mu$ g/DMEM 500  $\mu$ l/well.

[0051] 1/5,000 bleomycin group: bleomycin 500 ng/DMEM 500  $\mu$ l/well.

[0052] Medium group: DMEM

[0053] The above sample solution was added to mouse colon carcinoma cell CT26 prepared on a 6-well plate. The plate was kept for 30 minutes in an incubator at  $37^{\circ}$  C., and then the medium was exchanged with 500  $\mu$ l medium (DMEM containing 10% FCS). The cells were incubated for 2 days in a  $CO_2$  incubator at  $37^{\circ}$  C. Two days later, the number of viable cells was counted to evaluate the anticancer effect.

[0054] The results are shown in the following Table and in FIG. 1.

TABLE 1

Administration group	Number of mice	Mean	Standard deviation
1/50 Bleomycin group	2	81800	16688
1/500 Bleomycin group	2	164600	13859
1/5,000 Bleomycin group	2	196800	15274
HVJ-bleomycin group	2	16800	170
HVJ-PBS group	2	201100	8627
Medium group	2	220100	23617

[0055] The numbers of viable cells on average in the medium group and HVJ-PBS group were 220,100 and 201,100 respectively. The numbers of viable cells in the groups given bleomycin added to the medium (500 ng, 5  $\mu$ g, 50  $\mu$ g) were 196,800, 164,600 and 81,800 respectively, while the number of viable cells in the bleomycin HVJ-E incorporation group was 16,800. In percentage relative to the medium group (=100%), the degree of viable cells in the HVJ-PBS group was 91.4%, and the degrees of viable cells in the groups given bleomycin (500 ng, 5  $\mu$ g and 50  $\mu$ g) were 89.4%, 74.8% and 33.9% respectively, while the degree of viable cells in the bleomycin HVJ-E incorporation group was as low as 7.6%.

[0056] As a result, the dramatic cytotoxic effect could be achieved successfully by incorporation of bleomycin into HVJ-E. The significant effect of bleomycin introduced directly by the HVJ-E vector into cells can be understood when it is taken into consideration that the cytotoxic effect of bleomycin added to the culture solution is not so high.

[0057] This result indicated that by incorporating, into HVJ-E vector, the anticancer agent causing a severe side effect upon systemic administration, the chemical can be delivered directly to affected cells of the patient.

##### Example 2

##### In vivo Experiment

[0058] HVJ-E vector (6,000 HAU/600  $\mu$ l) was rapidly dissolved by shifting the temperature from  $-80^{\circ}$  C. to  $34.5^{\circ}$

C. A microtube containing the sample was centrifuged at 15,000 rpm for 15 minutes at 4° C. to give the HVJ-E vector as a precipitate, and the supernatant was removed. The resulting precipitate was suspended in 60 µl bleomycin/PBS (40 mg/ml) solution. Further, 2 µl of 3% Triton-X100 was added thereto to the final concentration of 0.1%, which was then kept on ice for 15 minutes. Thereafter, 500 µl PBS solution was added thereto. The microtube was centrifuged at 15,000 rpm for 15 minutes at 4° C., and the supernatant was removed without removing the precipitate, and 500 µl PBS solution was added thereto. The microtube was centrifuged again at 15,000 rpm for 15 minutes at 4° C., and the supernatant was removed without removing the precipitate. The resulting precipitate was suspended in 120 µl PBS.

[0059] The following administration groups were prepared and were compared to evaluate their effect.

[0060] HVJ-bleomycin administration group: 5,000 HAU, bleomycin 6.5 µg/100 µl/mouse

[0061] HVJ-PBS administration group: 5,000 HAU/100 µl/mouse

[0062] 65 µg/ml bleomycin administration group: bleomycin/PBS (65 µg/ml), 100 µl/mouse

[0063] PBS administration group: 100 µl PBS

[0064] In this animal experiment, BALB/c mice (8-week-old, male) were used. The site in which the cancer cell colon carcinoma CT26 was transplanted was a subcutaneous region in the back of a mouse, and hair on the back was shaved for measuring the volume of the transplanted cancer cells. The CT-26 cells to be transplanted were suspended in DMEM medium containing 10% FCS, and  $5 \times 10^6$  cells (100 µl PBS/mouse) were transplanted in the back. The mice were anesthetized by intraperitoneal administration of 500 µl of 20-fold-diluted Nembutal injection. The volume of the transplanted cancer cells was estimated by calculation of major axis  $\times$  minor axis  $\times$  minor axis/2. When the diameter of the tumor reached 7 to 8 mm one week after transplantation, 100 µl of the sample prepared above was administered into the site of the cancer in the mouse. On Days 7, 10, 13, 16 and 19 (that is, 3-day intervals) after transplantation of the cancer cells, the tumor diameter was measured to evaluate the anticancer effect. The number of animals was 8 in each group.

[0065] The results are shown in the following table and in FIG. 2. (Upper, mean; lower, standard deviation)

TABLE 2

	Number of days after administration			
	7	10	13	16
65 mg/ml bleomycin	158.4 25.4	413.70 71.20	754.7 206.6	1234.6 332.8
HVJ-bleomycin	136.2 16.2	285.70 77.60	456.7 116.4	676.1 209.2
HVJ-PBS	164.3 23.8	362.20 73.70	688.1 143.7	1083.1 243.8
Medium (PBS)	158.7 33.3	418.20 62.50	738.7 97.9	1277.7 162.7

[0066] The average tumor volume in each group on Day 16 after administration, and the rate of change relative to the medium (PBS) group are shown in FIG. 3.

[0067] In the tumor diameter with no difference recognized at the time of inoculation with the prepared sample, a difference was recognized on and after Day 3 (corresponding to Day 10 in FIG. 2) after administration of the sample (FIG. 2). On Day 9 (corresponding to Day 16 in FIG. 2, that is, 9 days after administration of the sample), the volumes of the tumor, calculated by the equation above, were 1,277 mm<sup>3</sup> in the PBS administration group, 1,235 mm<sup>3</sup> in the 65 µg/ml BLM administration group, 1,083 mm<sup>3</sup> in the HVJ-PBS administration group and 676 mm<sup>3</sup> in the HVJ-BLM administration group respectively (FIG. 2). When expressed in percentage, the PBS administration group: 65 µg/ml BLM administration group: HVJ-PBS administration group: HVJ-BLM administration group=0%:3.4%:15.2%:47.1% (FIG. 3). In the group with bleomycin administered directly to the tumor-affected area, the tumor shrinkage effect was as low as 3.4% relative to the effect (0%) of the PBS administration group, and the shrinkage effect on tumor volume was hardly recognized. Whether this is due to the direct administration into the tumor as opposed to usually conducted systemic administration or due to administration at low concentration, or due to another factor, cannot be judged from the present results. The tumor volume shrinkage effect in the HVJ-PBS administration group was 15.2%, thus indicating that even the HVJ-E vector only attains a certain effect. This anticancer effect at a certain extent is possibly due to the immune action induced by HVJ-E. On the other hand, the tumor volume in the incorporated bleomycin administration group was 47.1%, indicating a high antitumor effect.

[0068] This Example has revealed:

[0069] Antitumor effect was hardly recognized in vivo by direct administration of bleomycin to the solid tumor cells.

[0070] Even the HVJ-E vector only was recognized to attain a weak antitumor effect.

[0071] When bleomycin is incorporated into HVJ-E and administered directly into the solid tumor, an excellent antitumor effect was recognized.

### Example 3

#### In vivo Experiment (2)

##### (1) Test Design

[0072] Mouse colon cancer-derived CT-26 cells were transplanted subcutaneously into the backs of 8-week-old BALB/cAnNCrj male mice, and 0.2 or 0.4 mg Platocin injection (cisplatin, CDDP)/body was once administered intraperitoneally into the animals (10 animals in each group) wherein the diameter of the tumor (major axis) reached about 5 mm, and on Day 1 after administration, HVJ-E, or HVJ-E/BLM containing 13.2 mg bleomycin, was administered once to the tumor. On Day 21 after administration into the tumor, the mice were sacrificed to examine the antitumor action of HVJ-E/BLM.

[0073] The constitution of the group in this test is shown below.

Group	Intraperitoneal administration of the comparative control substance (mg/body)*		Administration of the test substance into the tumor (mg/tumor)**	
Control group	Physiological saline	0	Physiological saline	0
HVJ-E group	Physiological saline	0	HVJ-E	0
13 mg/tumor HVJ-E/BLM group	Physiological saline	0	HVJ-E/BLM	13
0.2 mg/body CDDP group	CDDP	0.2	Physiological saline	0
0.4 mg/body CDDP group	CDDP	0.4	Physiological saline	0
0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group	CDDP	0.2	HVJ-E/BLM	13
0.4 mg/body CDDP-HVJ-E group	CDDP	0.4	HVJ-E	0

\*amount of cisplatin (CDDP)

\*\*amount of bleomycin (BLM)

## (2) Experimental Methods

### 2-1) Culture of Cancer Cells

[0074] Mouse colon cancer-derived CT-26 cells were cultured in DMEM medium containing 10% FBS at 37° C. in the presence of 5% CO<sub>2</sub>.

[0075] The cells were cultured in a 75-cm<sup>2</sup> flask. The cells upon becoming about 80% confluent were subjected to subculture. After the DMEM (containing 10% FBS) was removed, the cells were washed with 10 mL phosphate-buffered physiological saline (PBS), and then the cells were released at 37° C. by adding 1 mL PBS containing 0.25% trypsin and 1 mmol/L EDTA-2Na. After 9 mL DMEM medium was added, the cells were collected and centrifuged (1000 rpm, 5 minutes), to recover the cells. After the supernatant was removed, the cells were diluted with DMEM medium containing 10% FBS and then cultured.

### 2-2) Preparation of a Suspension of the Cancer Cells

[0076] After the culture was removed from the cells which had become about 80% confluent, the culture flask was washed with PBS. A small amount of PBS containing 0.25% trypsin and 1 mmol/L EDTA-2Na was added and the flask was kept at 37° C. until release of the cells was initiated. The cells were collected with DMEM medium and centrifuged (1000 rpm, 5 minutes). After removal of the supernatant, the cells were suspended in PBS. The resulting suspension was centrifuged again (1000 rpm, 5 minutes). After the supernatant was removed, the residue was adjusted to 5×10<sup>7</sup> cells/ml with PBS.

### 2-3) Habituation of Mice

[0077] In the inspection and habituation period of 16 days, solid feed and drinking water were given freely.

### 2-4) Inoculation with the Cancer Cells

[0078] After the inspection and habituation were finished, hair of the animals was shaved by using hair clippers. Said cells (100 μL/site, 5×10<sup>6</sup> cells/body) were administered intracutaneously into the backs of 59 mice by using a disposal syringe and needle (26G). On the next day of

administration, the cells were administered in the same manner into 57 animals (animals not administered).

### 2-5) Grouping of the Animals

[0079] The tumor diameter (major axis, minor axis) was measured on Days 4, 5, 6 and 7 after transplantation (measurement was not conducted after grouping). Animals with a tumor diameter (major axis) of 4.5 to 5.5 mm (actual measurement of 4.64 to 5.48 mm) were grouped by stratified randomization such that the average tumor diameter (major axis) became almost the same in each group.

### 2-6) Administration

[0080] Using a disposal syringe and needle, the control substance (1000 μL) was once administered intraperitoneally into each group, and 1 day later, the test substance (100 μL) was administered into the tumor.

### 2-7) Measurement of Tumor Diameter

[0081] The administration day was regarded as Day 0 after administration. On Days 3, 6, 9, 12, 15, 18 and 21 after administration, the tumor diameters in each mouse was measured and the tumor volume (major axis×minor axis×minor axis÷2) was calculated.

### 2-8) Measurement of Tumor Weight

[0082] All mice in each group (after fasting for 16 to 24 hours) on Day 21 after administration were subjected to euthanasia by exsanguination under anesthesia by intraperitoneal administration of an aqueous solution (6.48 mg/mL, 5 mL/kg) of pentobarbital sodium (Tokyo Kasei Kogyo Co., Ltd.), and then the tumor was excised and measured for its weight.

## (3) Results

### 3-1) Tumor Volume

[0083] In the control group, HVJ-E group, 13 mg/tumor HVJ-E/BLM group, 0.2 mg/body CDDP group, 0.4 mg/body CDDP group and 0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group, the tumor volume was increased with time until Day 21 after administration, and the average tumor volumes on Day 21 were 3216.14, 2716.00, 2283.84, 1720.14, 1367.62 and 1022.34 mm<sup>3</sup> respectively in the above groups (3 mice in the 0.4 mg/body CDDP group, and 10 mice in each of the other groups). In one surviving mouse in the 0.4 mg/body CDDP-HVJ-E group, the tumor volume on Day 6 after administration was increased to 122.27 mm<sup>3</sup>, and the tumor volume was decreased to 118.82 mm<sup>3</sup> on Day 9 after administration, and further to 13.12-23.26 mm<sup>3</sup> on Day 12 to Day 21 after administration (see FIG. 4).

### 3-2) Tumor Weight

[0084] The tumor weights on Day 21 after administration were in the order of the control group (2570.35 mm)<sup>3</sup>≥HVJ-E group (2428.64 mg)>13 mg/tumor HVJ-E/BLM group (1680.65 mg)≥0.2 mg/body CDDP group (1619.79 mg)>0.4 mg/body CDDP group (1164.13 mg)>0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group (987.33 mg)>0.4 mg/body CDDP-HVJ-E group (90.4 mg). In the 0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group, the tumor present at the time of administration disappeared in 2 out of 10 mice.

[0085] The foregoing results revealed that the 13 mg/tumor HVJ-E/BLM group shows an antitumor action on the CT-26 cells transplanted in mice, and its action is enhanced by combined use of intraperitoneal administration of CDDP.

1. A pharmaceutical preparation comprising, as an active ingredient, a virus envelope vector having a chemotherapeutic agent incorporated therein.

2. The pharmaceutical preparation according to claim 1, wherein the chemotherapeutic agent is a cancerocidal agent, an anticancer agent, or an antitumor agent.

3. The pharmaceutical preparation according to claim 1, wherein the chemotherapeutic agent is at least one member selected from the group consisting of bleomycin and derivatives thereof, anthraquinone-based cancerocidal agents, mitomycin and derivatives thereof, actinomycin and derivatives thereof, taxane derivatives, camptothecin and derivatives thereof, cisplatin and derivatives thereof, staurosporine and derivatives thereof, vincristine, streptozotocin, 5-fluorouracil (5-FU) and derivatives thereof, viralbacin, dolastatin, and pharmacologically acceptable salts thereof.

4. The pharmaceutical preparation according to claim 1, wherein bleomycin and derivatives thereof are bleomycin or pharmacologically acceptable salt thereof or peplomycin or pharmacologically acceptable salts thereof.

5. The pharmaceutical preparation according to claim 1, wherein bleomycin and derivative thereof are bleomycin hydrochloride, bleomycin sulfate and peplomycin sulfate.

6. The pharmaceutical preparation according to claim 1, wherein the virus is derived from a virus belonging to a family selected from the group consisting of the retrovirus family, togavirus family, coronavirus family, flavivirus family, paramyxovirus family, orthomyxovirus family, bunyavirus family, rhabdovirus family, poxvirus family, herpes virus family, baculovirus family and hepadnavirus family.

7. The pharmaceutical preparation according to claim 1, wherein the virus is a member selected from the group consisting of Sendai virus, retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poxvirus and influenza virus.

8. The pharmaceutical preparation according to claim 1, wherein the chemotherapeutic agent is at least one member selected from the group consisting of bleomycin hydrochloride, bleomycin sulfate and peplomycin sulfate, and the virus is Sendai virus.

9. The pharmaceutical preparation according to claim 1, which is an injection.

10. The pharmaceutical preparation according to claim 1, wherein a surfactant is used in the step of incorporating the chemotherapeutic agent into the virus envelope vector.

11. The pharmaceutical preparation according to claim 10, wherein the surfactant is one member selected from the group consisting of Triton X100, deoxycholic acid and salts thereof, and cholic acid and salts thereof.

12. The pharmaceutical preparation according to claim 1, which is a therapeutic agent for solid cancer.

13. The pharmaceutical preparation according to claim 1, wherein the solid tumor is one member selected from the group consisting of lung cancer, breast cancer, digestive organ cancer, head and neck cancer, gynecologic cancer, urologic cancers, soft tissue and bone sarcoma, malignant lymphoma and cancer of unknown primary.

14. The pharmaceutical preparation according to claim 13, wherein the digestive organ cancer is one member selected from the group consisting of stomach cancer, colon cancer and esophagus cancer.

15. The pharmaceutical preparation according to claim 13, wherein the head and neck cancer is one member selected from the group consisting of upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, larynx cancer and oral cavity cancer.

16. The pharmaceutical preparation according to claim 13, wherein the gynecologic cancer is one member selected from the group consisting of uterus cancer, ovarian cancer and uterine cervical cancer.

17. The pharmaceutical preparation according to claim 13, wherein the urologic cancers is prostate cancer.

18. A method of treating a cancer, which comprises using a chemotherapeutic agent-incorporated virus envelope vector in combination with a platinum complex and/or an antimetabolite.

19. The method of treating a cancer according to claim 18, wherein the platinum complex is one member selected from the group consisting of cisplatin, carboplatin, Paraplatin and nedaplatin.

20. The method of treating a cancer according to claim 18, wherein the antimetabolite is one member selected from the group consisting of 6-mercaptopurine riboside, enocitabin, gemcitabine HCl, carmofur, cytarabine, cytarabine ocfosfate, tegafur, tegafur-uracil, tegafur-gimeracil-oteracil-potassium, doxifluridine, hydroxycarbamide, fluorouracil, methotrexate, mercaptopurine and fludarabine phosphate.

21. A method of treating a cancer, which comprises using a chemotherapeutic agent-incorporated virus envelope vector in combination with cisplatin and/or fluorouracil.

22. A method of treating a cancer, which comprises using a chemotherapeutic agent-incorporated virus envelope vector in combination with cisplatin and/or fluorouracil, and subsequent irradiation with radiations.

23. A method of treating a solid cancer, which comprises using a bleomycin- or its pharmacologically acceptable salt-incorporated Sendai virus envelope vector in combination with cisplatin and/or fluorouracil, and subsequent irradiation with radiations.

\* \* \* \* \*

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(54) Title: MEDICINAL PREPARATION HAVING CHEMOTHERAPEUTIC ENCAPSULATED THEREIN

(54) 発明の名称: 化学療法剤を封入した医薬製剤

(57) Abstract: It is intended to provide a medicinal preparation for transferring a chemotherapeutic (preferably an anticancer agent) into cells or the living body by using a virus envelope vector. Namely, a medicinal preparation containing a virus envelope vector having a chemotherapeutic encapsulated therein as the active ingredient. Examples of the anticancer agent include bleomycins, anthraquinone carcinostatic agents, mitomycins, actinomycins, taxane derivatives, camptothecins, cisplatin, staurosporines, vincristine, streptozotocin, 5-fluorouracil (5-FU) and its derivatives, pirarubicin, dolastatin and pharmacologically acceptable salts thereof. Examples of the virus include sendai virus, retro virus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, pox virus, influenza virus and so on.

(57) 要約: 本発明は、ウイルスエンベロープベクターを用いて細胞内あるいは生体内へ、化学療法剤、好ましくは抗癌剤を移入するための医薬製剤を提供する。化学療法剤を封入したウイルスエンベロープベクターを有効成分として含有する医薬製剤。抗癌剤として例えばブレオマイシン類、アントラキノン系制癌剤、マイトマイシン類、アクチノマイシン類、タキサン誘導体、カンプトテシン類、シスプラチン類、スタウロスポリン類、ビンクリスチン、ストレプトゾトシン、5-フルオロウラシル(5-FU)およびその誘導体、ビラルビシン、ドラスタチン(Dolastatin)およびそれらの薬理的に許容される塩を挙げることができる。ウイルスとして例えばセンダイウイルス、レトロウイルス、アデノウイルス、アデノ随伴ウイルス、ヘルペスウイルス、ワクシニアウイルス、ポックスウイルス、インフルエンザウイルス等を挙げることができる。

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MEDICINAL PREPARATION HAVING CHEMOTHERAPEUTIC ENCAPSULATED  
THEREIN

This application is the US national phase of international application PCT/JP2003/013860 filed 29 October 2003, which designated the U.S. and claims priority of JP 2002-320577, filed 1 November 2002, the entire contents of each of which are hereby incorporated by reference.

Technical Field

The present invention relates to a pharmaceutical preparation used in transfection of cells or living organisms with a chemotherapeutic agent by a gene delivery vector.

Background Art

It is said that the cure rate in cancer treatment is about 50% at present, and generally, such cure is brought about often by topical therapy such as surgical therapy and radiotherapy. It is in a very low rate that chemotherapy as systemic therapy can contribute solely to cure, particularly in treatment of solid tumor, and usually chemotherapy is used in combination with various therapies.

On the other hand, surgical therapy enables surgery in every organ cancer and is considered to reach completion as therapy,

and no further improvement in cure rate can be expected. The treatment results of susceptible organ cancers by radiotherapy also arrive at an almost fixed rate, and no further improvement in cure rate can be expected as well.

Accordingly, no significant improvement in cancer cure rate by these therapies can be expected in the future, and the development of further excellent chemotherapy is essential for further improving the cancer cure rate of 50% at present to arrive at cure for cancer.

An purpose of an anticancer agent used in chemotherapy lies in cytocidal effect on cells having a high ability to grow, such as cancer cells, and its damage to normal cells particularly myeloid cells having a high cellular growth ability is significant, and as a result, severe pain is given to patients. This is because the transfer of the anticancer agent is due to systemic administration by an injection, and the anticancer agent reaches normal cells other than cancer cells, so that the normal cells are killed and homeostasis does not function.

At present, however, the effect of an anticancer agent administered alone is regarded to be approximately about 30%, and it is expected that genetic information analysis and study on genome proceed so that selection of a suitable anticancer agent feasible can be expected in the future, but it is said that the therapy with the anticancer agent at present results in higher side effects.

This is because normal cells are damaged by systemic administration of an anticancer agent. Accordingly, if cancer tissue-specific delivery of the anticancer agent and subsequent incorporation thereof into cancer cells can be established, an ideal system of delivering the anticancer agent can be realized. In addition, if incorporation of the anticancer agent into a vesicle is feasible, a therapeutic method that is specific for target organ and cell with less influence (side effect) on normal cells can be established. Further, this can lead to reassessment of anticancer agents whose development was abandoned because of their strong side effects.

#### Disclosure of the Invention

As a result of extensive study for solving the problem described above, the present inventors could complete a pharmaceutical preparation comprising, as an active ingredient, a virus envelope vector having a chemotherapeutic agent incorporated therein.

Accordingly, the present invention provides, for example, a pharmaceutical preparation comprising an anticancer agent or the like incorporated in e.g. an inactivated HVJ-E vector having an ability to incorporate a foreign gene.

The present invention relates to a pharmaceutical preparation used in transfection of cells or living organisms with a chemotherapeutic agent, preferably a cancerocidal agent, an



anticancer agent, or an antitumor agent (hereinafter referred to collectively as anticancer agent), by using a gene delivery vector. The present invention relates more specifically to a pharmaceutical preparation by which a highly toxic anticancer agent is transferred by a gene delivery vector to the living organism and allowed to reach a target organ or cell safely with a reduction in side effects.

#### Best Mode for Carrying Out the Invention

Hereinafter, the present invention is described in more detail.

The chemotherapeutic agent used in the present invention is not particularly limited insofar as it is a low-molecular compound acting directly on cells, and for example, "Seikagaku Jiten" (Dictionary of Biochemistry), third edition, published by Tokyo Kagaku Dojin, describes: "At present, the subject of chemotherapy, that is, therapy using chemical substances of highly selective toxicity, has spread not only to microbial infections but also to malignant tumors", and it goes without saying that antibacterial agents, antitumor agents etc. are contained in the chemotherapeutic agent.

Preferable examples of the chemotherapeutic agent in the present invention include a cancerocidal agent, an anticancer agent or an antitumor agent (hereinafter referred to collectively as anticancer agent), and specifically the anticancer agent includes

bleomycin and derivatives thereof, anthraquinone-based cancerocidal agents including adriamycin and daunomycin, mitomycin and derivatives thereof, actinomycin and derivatives thereof, taxane derivatives such as taxol, camptothecin and derivatives thereof such as irinotecan, cisplatin and derivatives thereof, staurosporine and derivatives thereof, vincristine, streptozotocin, 5-fluorouracil (5-FU) and derivatives thereof, viralbicin and dolastatin, as well as pharmacologically acceptable salts thereof.

Preferable among these chemotherapeutic agents are bleomycin and derivatives thereof, and specific examples include bleomycin or pharmacologically acceptable salts thereof, or peplomycin or pharmacologically acceptable salts, more specifically bleomycin hydrochloride, bleomycin sulfate and peplomycin sulfate.

When the pharmaceutical preparation of the present invention is used as an anticancer agent, the type of cancer to which it is applicable is not particularly limited, and specifically, solid cancer, blood cell cancer, and the like can be exemplified. Among these cancers, the solid cancer is a preferable subject to which the pharmaceutical preparation is applicable.

Specific examples of the solid cancer include lung cancer, breast cancer, digestive organ cancer, head and neck cancer, gynecologic cancer, urologic cancer, soft tissue and bone sarcoma, malignant lymphoma, cancers of unknown primary etc., and more

specifically, for digestive organ cancers, stomach cancer, colon cancer, and esophagus cancer are exemplified, for head and neck cancers, upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, larynx cancer, and oral cavity cancer are exemplified, for gynecologic cancers, uterus cancer, ovarian cancer, and uterine cervical cancer are exemplified, and for urologic cancers, prostate cancer is exemplified.

Among these solid cancers, more preferable subjects include skin cancer, skin malignant tumor, head and neck cancers (upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, oral cavity cancer, and the like), lung cancer (particularly primary and metastasis flat epithelial cancer), esophagus cancer, malignant lymphomas (reticulum sarcoma, lymphosarcoma, Hodgkin's disease, and the like), uterine cervical cancer, neuroglioma, thyroid cancer, and prostate cancer.

The virus envelope vector in the present invention is a virus envelope which is prepared by removing RNA or DNA from virus. It is usually utilized to incorporate a gene, polynucleotide, oligonucleotide, plasmid or the like thereinto, for transfection.

The type of the virus is not limited, and specifically the virus includes, for example, viruses belonging to a family selected from the group consisting of the retrovirus family, togavirus family, coronavirus family, flavivirus family, paramyxovirus family, orthomyxovirus family, bunyavirus family, rhabdovirus family,

poxvirus family, herpes virus family, baculovirus family, and hepadna virus family.

Specifically, the virus in the present invention includes, for example, Sendai virus, retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poxvirus, influenza virus, and the like.

Among these viruses, Sendai virus (hemagglutinating virus of Japan (referred to hereinafter as HVJ)) that is one of mouse pneumonia viruses can be mentioned as a preferable example.

Specifically, Sendai virus, for example, VR-105 and VR-907 can be purchased from American Type Culture Collection (ATCC, telephone 1-703-365-2700, P.O. Box 1549, Manassas, VA 20108, USA.

The virus envelope vector is described in more detail in, for example, JP-A2001-286282 (WO01/57204), JP-A2002-065278, WO-A 03/014338 (PCT/JP02/07879), and can be prepared specifically according to e.g. Example 8 in JP-A 2001-286282.

In the step of incorporating a chemotherapeutic agent in the virus envelope vector, a surfactant is preferably used, and specific examples of the surfactant include Triton X100, deoxycholic acid or a salt thereof, and cholic acid or a salt thereof. Preferably, the salt of deoxycholic acid includes sodium deoxycholate, and preferably, the salt of cholic acid includes sodium cholate.

The form of the pharmaceutical preparation of the present invention is not limited, but its specific example is an injectable

solution, ointment, or the like, preferably an injectable solution.

Here, the present inventable solution is described in more detail by reference to the inactivated Sendai virus envelope vector (referred to hereinafter as HVJ-E vector).

When an anticancer agent is incorporated into the HVJ-E vector, the anticancer agent is dissolved in a buffer solution. The buffer solution used herein is not limited, and specifically, for example, TE buffer solution (10 mM Tris, 1 mM EDTA (pH 8.0)), PBS (phosphate buffer solution) can be suitably selected and used wherein the pH of the buffer solution is preferably 6 to 9.

A preferable feature of the present invention is that, in an *in vitro* experiment, an anticancer agent having a strong side effect or toxicity can be incorporated into HVJ-E vector to be delivered directly into cells without leakage of the anticancer agent into a culture solution.

In an *in vivo* animal experiment, not systemic administration but local administration of the anticancer agent is feasible, and the anticancer agent can be efficiently delivered into solid-cancer cells only.

Humans can be treated by chemotherapy of administering the anticancer agent-incorporated HVJ-E vector solely, or by locally administering it into progressive cancer patients whom the anticancer agent cannot be administered, to attain cancer regression, and simultaneously using radiotherapy and/or surgical

treatment to achieve further excellent anticancer effects.

In an *in vitro* experiment, host cells are transfected with the anticancer agent-incorporated HVJ-E vector. As the procedure in this case, for example, a method of adding a solution of the anticancer agent-incorporated HVJ-E vector to a medium for culturing the cells can be used.

The transfection is performed for about 30 minutes to 48 hours when it is carried out at 37°C. Judgment of the effect is conducted preferably by counting the number of viable cells or by WST assay (technique of counting viable cells; cell counting kit, Dojin Kagaku).

When the subject of an *in vivo* animal experiment is, for example, a mouse, it is preferable that a normal mouse that is not an immune-deficient mouse is used where the cancer cell is an isograft, and that a nude mouse or SCID mouse is used in the case of xenograft.

Cancer cells cultured in a Petri dish are transplanted subcutaneously to a mouse, and after growth of the transplanted cells, the anticancer agent-incorporated HVJ-E vector is administered into the grown solid cancer, and the major axis and minor axis of the cancer can be measured to determine the anticancer effect.

According to the present invention, there is provided a method capable of delivering an anticancer agent having a strong side effect to the site of a cancer easily and safely.

Accordingly, the HVJ-E vector can be used to enable new chemotherapy for any solid cancer, which is increasing rapidly in Japan, such as lung cancer, breast cancer, digestive organ cancers such as stomach cancer, colon cancer or esophagus cancer, head and neck cancers (upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, larynx cancer, oral cavity cancer etc.), gynecologic cancers (uterus cancer, ovarian cancer, uterine cervical cancer etc.), urologic cancers (prostate cancer), soft tissue and bone sarcoma, malignant lymphoma, cancers of unknown primary, and the like.

#### Brief Description of Drawings

Fig. 1 is a graph wherein the numbers (mean  $\pm$  standard deviation) of viable cells in the respective groups in an *in vitro* experiment are compared.

Fig. 2 is a graph wherein the average tumor volumes (mean  $\pm$  standard deviation) in the respective groups in an *in vivo* experiment are compared.

Fig. 3 is a graph showing a change in the average tumor volume (mean  $\pm$  standard deviation) in each group, relative to the medium (PBS) group, on Day 16 after administration in an *in vivo* experiment.

Fig. 4 is a graph showing the result of tumor volume in Example 3.

## Examples

Hereinafter, the present invention is described in more detail by reference to Examples, but the present invention is not limited by these Examples.

### Example 1. *In vitro* experiment

According to Example 8 in JP-A 2001-286282, 6,000 HAU/600  $\mu$ l (for six 6-well plates) of inactivated HVJ-E vector was allowed to have a change in temperature from  $-80^{\circ}\text{C}$  to  $34.5^{\circ}\text{C}$ . A microtube containing the sample was centrifuged at 15,000 rpm for 15 minutes at  $4^{\circ}\text{C}$  to give the HVJ-E vector as a precipitate, and the supernatant was removed. The resulting precipitate was suspended in 60  $\mu$ l bleomycin/PBS (5 mg/ml) (bleomycin HCl manufactured by Nippon Kayaku Co., Ltd.) solution. Further, 2  $\mu$ l of 3% Triton-X100 was added thereto to prepare a sample containing Triton-X100 at a final concentration of 0.1%, which was then left on ice for 15 minutes. Thereafter, 500  $\mu$ l PBS solution was added thereto. The microtube was centrifuged at 15,000 rpm for 15 minutes at  $4^{\circ}\text{C}$ , and the supernatant was removed without removing the precipitate, and 500  $\mu$ l PBS solution was added again to the precipitate. The microtube was centrifuged again at 15,000 rpm for 15 minutes at  $4^{\circ}\text{C}$ , and the supernatant was removed without removing the precipitate.

The resulting precipitate was suspended in 180  $\mu$ l PBS, and the resulting sample solution was pipetted in a volume of 30



µl/microtube into 6 microtubes. Five microliters of protamine sulfate solution prepared at 5 mg/ml and 500 µl DMEM solution (Dulbecco's modified Eagle medium) were added to each tube.

As the administration group, the followings were prepared and compared to evaluate their effect.

HVJ-bleomycin group; 1,000 HAU, bleomycin 200 ng/DMEM 500 µl/well.

HVJ-PBS group: 1,000 HAU/DMEM 500 µl/well.

1/50 bleomycin group: bleomycin 50 µg/DMEM 500 µl/well.

1/500 bleomycin group: bleomycin 5 µg/DMEM 500 µl/well.

1/5,000 bleomycin group: bleomycin 500 ng/DMEM 500 µl/well.

Medium group: DMEM

The above sample solution was added to mouse colon carcinoma cell CT26 prepared on a 6-well plate. The plate was kept for 30 minutes in an incubator at 37°C, and then the medium was exchanged with 500 µl medium (DMEM containing 10% FCS). The cells were incubated for 2 days in a CO<sub>2</sub> incubator at 37°C. Two days later, the number of viable cells was counted to evaluate the anticancer effect.

The results are shown in the following Table and in Fig. 1.

Table 1

Administration group	Number of mice	Mean	Standard deviation
1/50 Bleomycin group	2	81800	16688
1/500 Bleomycin group	2	164600	13859
1/5,000 Bleomycin group	2	196800	15274
HVJ-bleomycin group	2	16800	170
HVJ-PBS group	2	201100	8627
Medium group	2	220100	23617

The numbers of viable cells on average in the medium group and HVJ-PBS group were 220,100 and 201,100 respectively. The numbers of viable cells in the groups given bleomycin added to the medium (500 ng, 5  $\mu$ g, 50  $\mu$ g) were 196,800, 164,600 and 81,800 respectively, while the number of viable cells in the bleomycin HVJ-E incorporation group was 16,800. In percentage relative to the medium group (= 100%), the degree of viable cells in the HVJ-PBS group was 91.4%, and the degrees of viable cells in the groups given bleomycin (500 ng, 5  $\mu$ g and 50  $\mu$ g) were 89.4%, 74.8% and 33.9% respectively, while the degree of viable cells in the bleomycin HVJ-E incorporation group was as low as 7.6%.

As a result, the dramatic cytotoxic effect could be achieved successfully by incorporation of bleomycin into HVJ-E. The significant effect of bleomycin introduced directly by the HVJ-E vector into cells can be understood when it is taken into consideration that the cytotoxic effect of bleomycin added to the culture solution is not so high.

This result indicated that by incorporating, into HVJ-E vector,

the anticancer agent causing a severe side effect upon systemic administration, the chemical can be delivered directly to affected cells of the patient.

#### Example 2. *In vivo* experiment

HVJ-E vector (6,000 HAU/600  $\mu$ l) was rapidly dissolved by shifting the temperature from  $-80^{\circ}\text{C}$  to  $34.5^{\circ}\text{C}$ . A microtube containing the sample was centrifuged at 15,000 rpm for 15 minutes at  $4^{\circ}\text{C}$  to give the HVJ-E vector as a precipitate, and the supernatant was removed. The resulting precipitate was suspended in 60  $\mu$ l bleomycin/PBS (40 mg/ml) solution. Further, 2  $\mu$ l of 3% Triton-X100 was added thereto to the final concentration of 0.1%, which was then kept on ice for 15 minutes. Thereafter, 500  $\mu$ l PBS solution was added thereto. The microtube was centrifuged at 15,000 rpm for 15 minutes at  $4^{\circ}\text{C}$ , and the supernatant was removed without removing the precipitate, and 500  $\mu$ l PBS solution was added thereto. The microtube was centrifuged again at 15,000 rpm for 15 minutes at  $4^{\circ}\text{C}$ , and the supernatant was removed without removing the precipitate. The resulting precipitate was suspended in 120  $\mu$ l PBS.

The following administration groups were prepared and were compared to evaluate their effect.

HVJ-bleomycin administration group: 5,000 HAU, bleomycin 6.5  $\mu\text{g}/100$   $\mu$ l/mouse

HVJ-PBS administration group: 5,000 HAU/100 µl/mouse

65 µg/ml bleomycin administration group: bleomycin/PBS (65 µg/ml),  
100 µl/mouse

PBS administration group: 100 µl PBS

In this animal experiment, BALB/c mice (8-week-old, male) were used. The site in which the cancer cell colon carcinoma CT26 was transplanted was a subcutaneous region in the back of a mouse, and hair on the back was shaved for measuring the volume of the transplanted cancer cells. The CT-26 cells to be transplanted were suspended in DMEM medium containing 10% FCS, and  $5 \times 10^6$  cells (100 µl PBS/mouse) were transplanted in the back. The mice were anesthetized by intraperitoneal administration of 500 µl of 20-fold-diluted Nembutal injection. The volume of the transplanted cancer cells was estimated by calculation of  $\text{major axis} \times \text{minor axis} \times \text{minor axis} / 2$ . When the diameter of the tumor reached 7 to 8 mm one week after transplantation, 100 µl of the sample prepared above was administered into the site of the cancer in the mouse. On Days 7, 10, 13, 16 and 19 (that is, 3-day intervals) after transplantation of the cancer cells, the tumor diameter was measured to evaluate the anticancer effect. The number of animals was 8 in each group.

The results are shown in the following table and in Fig. 2.  
(Upper, mean; lower, standard deviation)

Table 2

Number of days after administration	7	10	13	16
65 mg/ml bleomycin	158.4	413.70	754.7	1234.6
	25.4	71.20	206.6	332.8
HVJ-bleomycin	136.2	285.70	456.7	676.1
	16.2	77.60	116.4	209.2
HVJ-PBS	164.3	362.20	688.1	1083.1
	23.8	73.70	143.7	243.8
Medium (PBS)	158.7	418.20	738.7	1277.7
	33.3	62.50	97.9	162.7

The average tumor volume in each group on Day 16 after administration, and the rate of change relative to the medium (PBS) group are shown in Fig. 3.

In the tumor diameter with no difference recognized at the time of inoculation with the prepared sample, a difference was recognized on and after Day 3 (corresponding to Day 10 in Fig. 2) after administration of the sample (Fig. 2). On Day 9 (corresponding to Day 16 in Fig. 2, that is, 9 days after administration of the sample), the volumes of the tumor, calculated by the equation above, were 1,277 mm<sup>3</sup> in the PBS administration group, 1,235 mm<sup>3</sup> in the 65 µg/ml BLM administration group, 1,083 mm<sup>3</sup> in the HVJ-PBS administration group and 676 mm<sup>3</sup> in the HVJ-BLM administration group respectively (Fig. 2). When expressed in percentage, the PBS administration group : 65 µg/ml BLM administration group : HVJ-PBS administration group : HVJ-BLM administration group = 0% : 3.4% : 15.2% : 47.1% (Fig. 3). In

the group with bleomycin administered directly to the tumor-affected area, the tumor shrinkage effect was as low as 3.4% relative to the effect (0%) of the PBS administration group, and the shrinkage effect on tumor volume was hardly recognized. Whether this is due to the direct administration into the tumor as opposed to usually conducted systemic administration or due to administration at low concentration, or due to another factor, cannot be judged from the present results. The tumor volume shrinkage effect in the HVJ-PBS administration group was 15.2%, thus indicating that even the HVJ-E vector only attains a certain effect. This anticancer effect at a certain extent is possibly due to the immune action induced by HVJ-E. On the other hand, the tumor volume in the incorporated bleomycin administration group was 47.1%, indicating a high antitumor effect.

This Example has revealed:

- Antitumor effect was hardly recognized *in vivo* by direct administration of bleomycin to the solid tumor cells.
- Even the HVJ-E vector only was recognized to attain a weak antitumor effect.
- When bleomycin is incorporated into HVJ-E and administered directly into the solid tumor, an excellent antitumor effect was recognized.

Example 3. *In vivo* experiment (2)

### (1) Test Design

Mouse colon cancer-derived CT-26 cells were transplanted subcutaneously into the backs of 8-week-old BALB/cAnNCrj male mice, and 0.2 or 0.4 mg Platocin injection (cisplatin, CDDP)/body was once administered intraperitoneally into the animals (10 animals in each group) wherein the diameter of the tumor (major axis) reached about 5 mm, and on Day 1 after administration, HVJ-E, or HVJ-E/BLM containing 13.2 mg bleomycin, was administered once to the tumor. On Day 21 after administration into the tumor, the mice were sacrificed to examine the antitumor action of HVJ-E/BLM.

The constitution of the group in this test is shown below.

Group	Intraperitoneal administration of the comparative control substance		Administration of the test substance into the tumor	
	(mg/body)*		(mg/tumor)**	
Control group	Physiological saline	0	Physiological saline	0
HVJ-E group	Physiological saline	0	HVJ-E	0
13mg/tumor HVJ-E/BLM group	Physiological saline	0	HVJ-E/BLM	13
0.2mg/body CDDP group	CDDP	0.2	Physiological saline	0
0.4mg/body CDDP group	CDDP	0.4	Physiological saline	0
0.2mg/body CDDP-13mg / tumor HVJ-E/BLM group	CDDP	0.2	HVJ-E/BLM	13
0.4mg/body CDDP-HVJ-E group	CDDP	0.4	HVJ-E	0

\*: amount of cisplatin (CDDP)

\*\* : amount of bleomycin (BLM)

## (2) Experimental Methods

### 2-1) Culture of cancer cells

Mouse colon cancer-derived CT-26 cells were cultured in DMEM medium containing 10% FBS at 37°C in the presence of 5% CO<sub>2</sub>.

The cells were cultured in a 75-cm<sup>2</sup> flask. The cells upon becoming about 80% confluent were subjected to subculture. After the DMEM (containing 10% FBS) was removed, the cells were washed with 10 mL phosphate-buffered physiological saline (PBS), and then the cells were released at 37°C by adding 1 mL PBS containing 0.25% trypsin and 1 mmol/L EDTA-2Na. After 9 mL DMEM medium was added, the cells were collected and centrifuged (1000 rpm, 5 minutes), to recover the cells. After the supernatant was removed, the cells were diluted with DMEM medium containing 10% FBS and then cultured.

### 2-2) Preparation of a suspension of the cancer cells

After the culture was removed from the cells which had become about 80% confluent, the culture flask was washed with PBS. A small amount of PBS containing 0.25% trypsin and 1 mmol/L EDTA-2Na was added and the flask was kept at 37°C until release of the cells was initiated. The cells were collected with DMEM medium and centrifuged (1000 rpm, 5 minutes). After removal of the supernatant, the cells were suspended in PBS. The resulting suspension was centrifuged again (1000 rpm, 5 minutes). After the supernatant was removed, the residue was adjusted to  $5 \times 10^7$  cells/ml with PBS.



### 2-3) Habituation of mice

In the inspection and habituation period of 16 days, solid feed and drinking water were given freely.

### 2-4) Inoculation with the cancer cells

After the inspection and habituation were finished, hair of the animals was shaved by using hair clippers. Said cells (100  $\mu\text{L}/\text{site}$ ,  $5 \times 10^6$  cells/body) were administered intracutaneously into the backs of 59 mice by using a disposal syringe and needle (26G). On the next day of administration, the cells were administered in the same manner into 57 animals (animals not administered).

### 2-5) Grouping of the animals

The tumor diameter (major axis, minor axis) was measured on Days 4, 5, 6 and 7 after transplantation (measurement was not conducted after grouping). Animals with a tumor diameter (major axis) of 4.5 to 5.5 mm (actual measurement of 4.64 to 5.48 mm) were grouped by stratified randomization such that the average tumor diameter (major axis) became almost the same in each group.

### 2-6) Administration

Using a disposal syringe and needle, the control substance (1000  $\mu\text{L}$ ) was once administered intraperitoneally into each group, and 1 day later, the test substance (100  $\mu\text{L}$ ) was administered into the tumor.

### 2-7) Measurement of tumor diameter

The administration day was regarded as Day 0 after

administration. On Days 3, 6, 9, 12, 15, 18 and 21 after administration, the tumor diameters in each mouse was measured and the tumor volume (major axis×minor axis×minor axis÷2) was calculated.

#### 2-8) Measurement of tumor weight

All mice in each group (after fasting for 16 to 24 hours) on Day 21 after administration were subjected to euthanasia by exsanguination under anesthesia by intraperitoneal administration of an aqueous solution (6.48 mg/mL, 5 mL/kg) of pentobarbital sodium (Tokyo Kasei Kogyo Co., Ltd.), and then the tumor was excised and measured for its weight.

### (3) Results

#### 3-1) Tumor volume

In the control group, HVJ-E group, 13 mg/tumor HVJ-E/BLM group, 0.2 mg/body CDDP group, 0.4 mg/body CDDP group and 0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group, the tumor volume was increased with time until Day 21 after administration, and the average tumor volumes on Day 21 were 3216.14, 2716.00, 2283.84, 1720.14, 1367.62 and 1022.34 mm<sup>3</sup> respectively in the above groups (3 mice in the 0.4 mg/body CDDP group, and 10 mice in each of the other groups). In one surviving mouse in the 0.4 mg/body CDDP-HVJ-E group, the tumor volume on Day 6 after administration was increased to 122.27 mm<sup>3</sup>, and the tumor volume was decreased to 118.82 mm<sup>3</sup> on Day 9 after administration, and further to 13.12-23.26 mm<sup>3</sup> on Day 12 to Day

21 after administration (see Fig. 4).

### 3-2) Tumor weight

The tumor weights on Day 21 after administration were in the order of the control group (2570.35 mm)  $\geq$  HVJ-E group (2428.64 mg)  $>$  13 mg/tumor HVJ-E/BLM group (1680.65 mg)  $\geq$  0.2 mg/body CDDP group (1619.79 mg)  $>$  0.4 mg/body CDDP group (1164.13 mg)  $>$  0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group (987.33 mg)  $>$  0.4 mg/body CDDP-HVJ-E group (90.4 mg). In the 0.2 mg/body CDDP-13 mg/tumor HVJ-E/BLM group, the tumor present at the time of administration disappeared in 2 out of 10 mice.

The foregoing results revealed that the 13 mg/tumor HVJ-E/BLM group shows an antitumor action on the CT-26 cells transplanted in mice, and its action is enhanced by combined use of intraperitoneal administration of CDDP.

## Claims

1. A pharmaceutical preparation comprising, as an active ingredient, a virus envelope vector having a chemotherapeutic agent incorporated therein.

2. The pharmaceutical preparation according to claim 1, wherein the chemotherapeutic agent is a cancerocidal agent, an anticancer agent, or an antitumor agent.

3. The pharmaceutical preparation according to claim 1, wherein the chemotherapeutic agent is at least one member selected from the group consisting of bleomycin and derivatives thereof, anthraquinone-based cancerocidal agents, mitomycin and derivatives thereof, actinomycin and derivatives thereof, taxane derivatives, camptothecin and derivatives thereof, cisplatin and derivatives thereof, staurosporine and derivatives thereof, vincristine, streptozotocin, 5-fluorouracil (5-FU) and derivatives thereof, viralbicin, dolastatin, and pharmacologically acceptable salts thereof.

4. The pharmaceutical preparation according to claim 1, wherein bleomycin and derivatives thereof are bleomycin or pharmacologically acceptable salt thereof or peplomycin or pharmacologically acceptable salts thereof.

5. The pharmaceutical preparation according to claim 1, wherein bleomycin and derivative thereof are bleomycin

hydrochloride, bleomycin sulfate and peplomycin sulfate.

6. The pharmaceutical preparation according to claim 1, wherein the virus is derived from a virus belonging to a family selected from the group consisting of the retrovirus family, togavirus family, coronavirus family, flavivirus family, paramyxovirus family, orthomyxovirus family, bunyavirus family, rhabdovirus family, poxvirus family, herpes virus family, baculovirus family and hepadnavirus family.

7. The pharmaceutical preparation according to claim 1, wherein the virus is a member selected from the group consisting of Sendai virus, retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poxvirus and influenza virus.

8. The pharmaceutical preparation according to claim 1, wherein the chemotherapeutic agent is at least one member selected from the group consisting of bleomycin hydrochloride, bleomycin sulfate and peplomycin sulfate, and the virus is Sendai virus.

9. The pharmaceutical preparation according to claim 1, which is an injection.

10. The pharmaceutical preparation according to claim 1, wherein a surfactant is used in the step of incorporating the chemotherapeutic agent into the virus envelope vector.

11. The pharmaceutical preparation according to claim 10, wherein the surfactant is one member selected from the group consisting of Triton X100, deoxycholic acid and salts thereof,

and cholic acid and salts thereof.

12. The pharmaceutical preparation according to claim 1, which is a therapeutic agent for solid cancer.

13. The pharmaceutical preparation according to claim 1, wherein the solid tumor is one member selected from the group consisting of lung cancer, breast cancer, digestive organ cancer, head and neck cancer, gynecologic cancer, urologic cancers, soft tissue and bone sarcoma, malignant lymphoma and cancer of unknown primary.

14. The pharmaceutical preparation according to claim 13, wherein the digestive organ cancer is one member selected from the group consisting of stomach cancer, colon cancer and esophagus cancer.

15. The pharmaceutical preparation according to claim 13, wherein the head and neck cancer is one member selected from the group consisting of upper jaw cancer, tongue cancer, lip cancer, pharynx cancer, larynx cancer and oral cavity cancer.

16. The pharmaceutical preparation according to claim 13, wherein the gynecologic cancer is one member selected from the group consisting of uterus cancer, ovarian cancer and uterine cervical cancer.

17. The pharmaceutical preparation according to claim 13, wherein the urologic cancers is prostate cancer.

18. A method of treating a cancer, which comprises using a

chemotherapeutic agent-incorporated virus envelope vector in combination with a platinum complex and/or an antimetabolite.

19. The method of treating a cancer according to claim 18, wherein the platinum complex is one member selected from the group consisting of cisplatin, carboplatin, Paraplatin and nedaplatin.

20. The method of treating a cancer according to claim 18, wherein the antimetabolite is one member selected from the group consisting of 6-mercaptopurine riboside, enocitabin, gemcitabine HCl, carmofur, cytarabine, cytarabine ocfosphate, tegafur, tegafur-uracil, tegafur-gimeracil-oteracil-potassium, doxifluridine, hydroxycarbamide, fluorouracil, methotrexate, mercaptopurine and fludarabine phosphate.

21. A method of treating a cancer, which comprises using a chemotherapeutic agent-incorporated virus envelope vector in combination with cisplatin and/or fluorouracil.

22. A method of treating a cancer, which comprises using a chemotherapeutic agent-incorporated virus envelope vector in combination with cisplatin and/or fluorouracil, and subsequent irradiation with radiations.

23. A method of treating a solid cancer, which comprises using a bleomycin- or its pharmacologically acceptable salt-incorporated Sendai virus envelope vector in combination with cisplatin and/or fluorouracil, and subsequent irradiation with radiations.

## Abstract of the Disclosure

It is intended to provide a medicinal preparation for transferring a chemotherapeutic (preferably an anticancer agent) into cells or the living body by using a virus envelope vector. Namely, a medicinal preparation containing a virus envelope vector having a chemotherapeutic encapsulated therein as the active ingredient. Examples of the anticancer agent include bleomycins, anthraquinone carcinostatic agents, mitomycins, actinomycins, taxane derivatives, camptothecins, cisplatins, staurosporines, vincristine, streptozotocin, 5-fluorouracil (5-FU) and its derivatives, pirarubicin, dolastatin and pharmacologically acceptable salts thereof. Examples of the virus include Sendai virus, retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, pox virus, influenza virus and so on.



Fig. 1

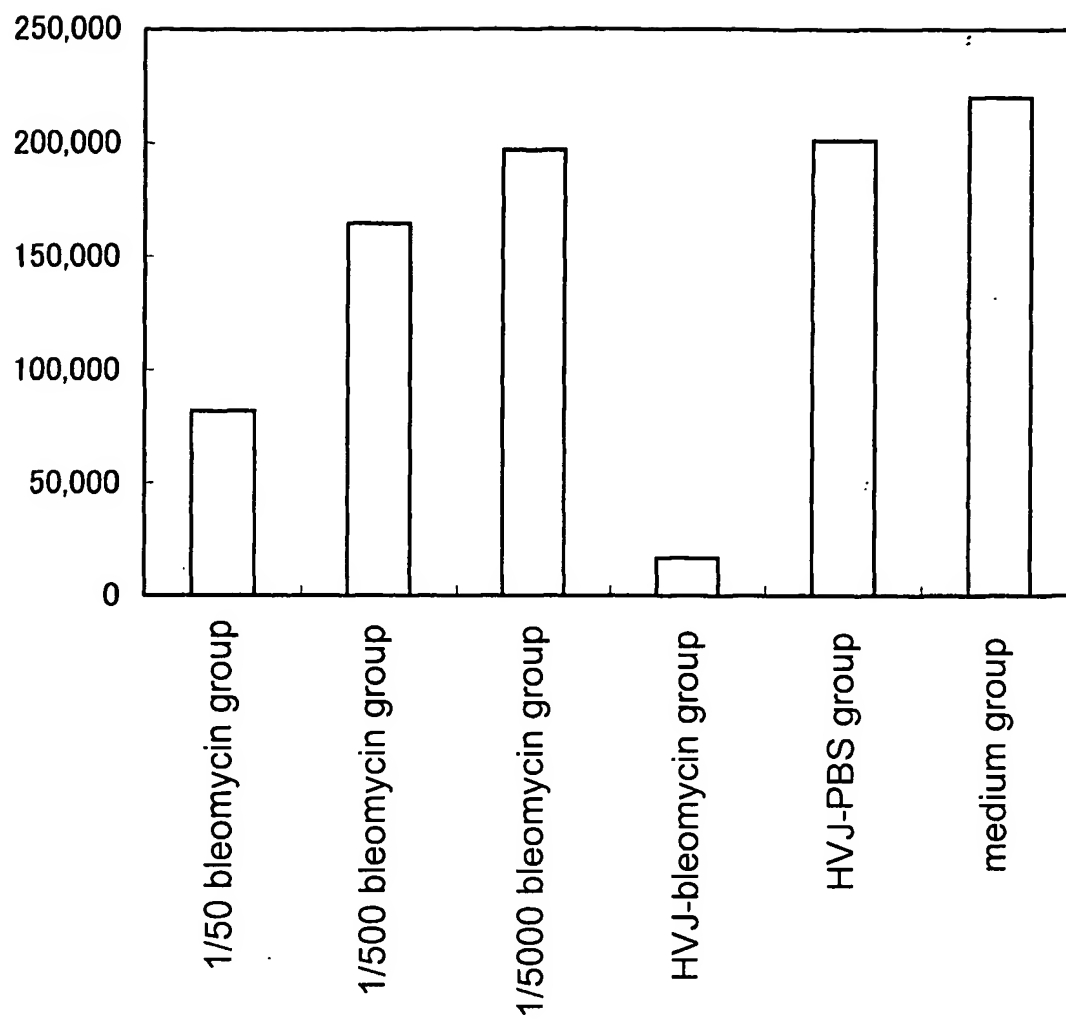


Fig. 2

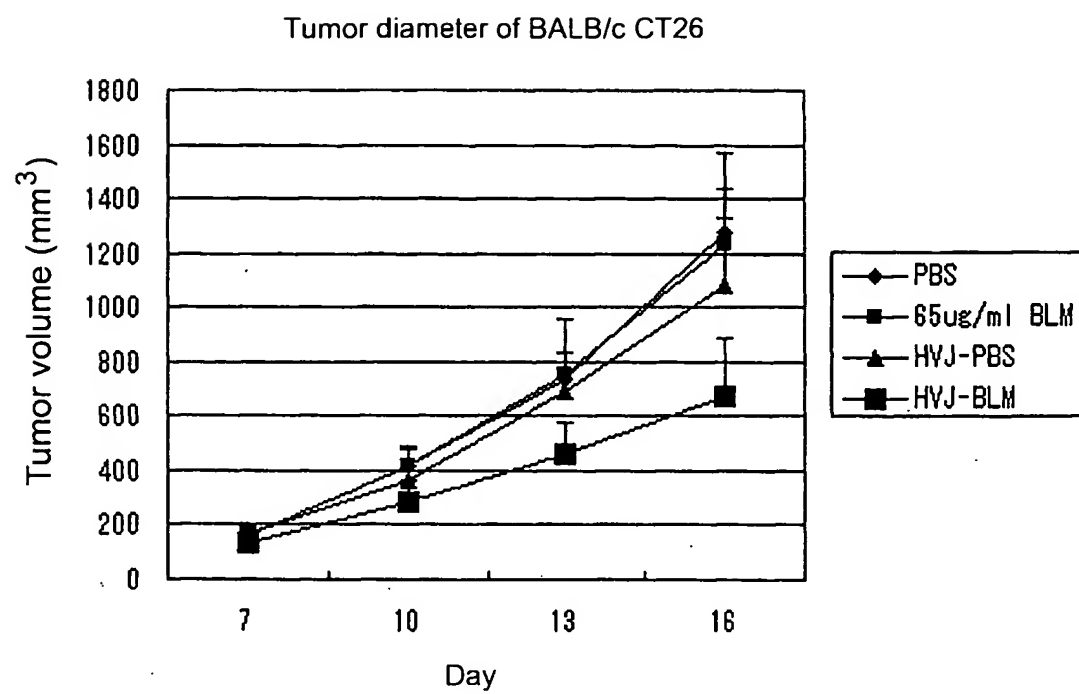


Fig. 3

Average tumor volume in each group on Day 16 after administration

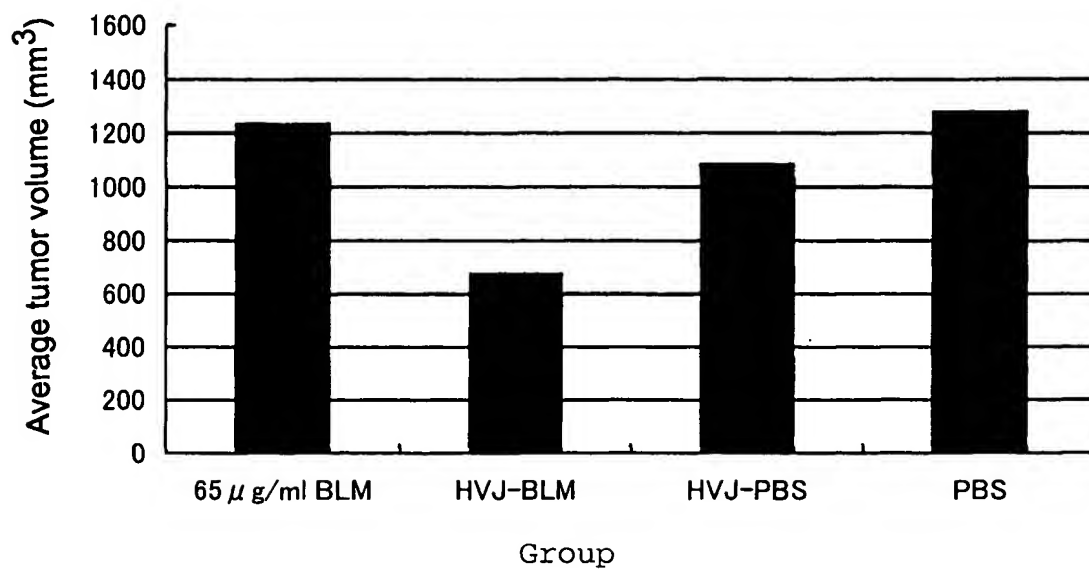


Fig.4

